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Ascertaining the biochemical function of an essential pectin methylesterase in the gut microbe *Bacteroides thetaiotaomicron*

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Pectins are a major dietary nutrient source for the human gut microbiota. The prominent gut microbe *Bacteroides thetaiotaomicron* was recently shown to encode the founding member (BT1017) of a new family of pectin methylesterases essential for the metabolism of the complex pectin rhamnogalacturonan-II (RG-II). However, biochemical and structural knowledge of this family is lacking. Here, we showed that BT1017 is critical for the metabolism of an RG-II-derived oligosaccharide Δ BT1017oligoB generated by a BT1017 deletion mutant (Δ BT1017) during growth on carbohydrate extract from apple juice. Structural analyses of Δ BT1017oligoB using a combination of enzymatic, mass spectrometric, and NMR approaches revealed that it is a bimethylated nonaoligosaccharide (GlcA- β 1,4-(2-O-Me-Xyl- α 1,3)-Fuc- α 1,4-(GalA- β 1,3)-Rha- α 1,3-Api- β 1,2-(Araf- α 1,3)-(GalA- α 1,4)-GalA) containing components of the RG-II backbone and its side chains. We showed that the catalytic module of BT1017 adopts an α/β -hydrolase fold, consisting of a central twisted 10-stranded β -sheet sandwiched by several α -helices. This constitutes a new fold for pectin methylesterases, which are predominantly right-handed β -helical proteins. Bioinformatic analyses revealed that the family is dominated by sequences from prominent genera of the human gut microbiota, including *Bacteroides* and *Prevotella*. Our results not only highlight the critical role played by this family of enzymes in pectin metabolism but also provide new insights into the molecular basis of the adaptation of *B. thetaiotaomicron* to the human gut.

The human large intestine is home to a large microbial community termed the human gut microbiota (HGM), which has substantial impact on the health and physiology of its host. Pectins, which are a major component of plant-based diets, have been shown to exert a significant selective pressure on HGM species (1–3) and hence have great potential as tools to manipulate the HGM. Pectins are defined as D-galacturonic acid-containing plant cell wall polysaccharides. The pectin macrostructure consists of

three major polysaccharides: rhamnogalacturonan-I (RG-I), rhamnogalacturonan-II (RG-II), and homogalacturonan (4). Of these, RG-II is the most complex, consisting of several heterogeneous side chains (A, B, C, D, E, and F), which are linked to a backbone of D-galacturonic acid (GalA) residues (Fig. 1A) (5). In total, RG-II contains at least 22 distinct glycosidic linkages and 13 different monosaccharides (Fig. 1A). The structure of RG-II is highly conserved; however, there is some variation in RG-II between plant species particularly at the termini of side chain B and in the methylation pattern of side chain A, as described previously (6, 7).

Bacteroides thetaiotaomicron is a prominent member of the HGM, equipped with a large repertoire of carbohydrate-active enzymes (CAZymes) and considered as a generalist being able to forage on a wide range of dietary or host glycans (for a review see Ref. 4). *B. thetaiotaomicron* has the ability to cleave 21 of the 22 glycosidic linkages in RG-II (except that in the disaccharide 2-O-Me-Xyl- α 1,3-Fuc) (5). Among the *B. thetaiotaomicron* repertoire, several founding members of novel CAZyme families were characterized including a pectin methylesterase (PME) BT1017. BT1017 was shown to remove the 6-O-methyl decoration of GalA in the homogalacturonan backbone of RG-II, therefore playing a critical role in enabling access to the rest of the RG-II structure by other RG-II-degrading enzymes (5). Currently more than 18 carbohydrate esterase families have been assigned, according to the CAZyme database (8); this topic was recently reviewed by Nakamura *et al.* (9). Of the 18 families, CE8 is the only family that contains PMEs. BT1017, however, displays no sequence similarity to CE8 esterases, and hence the structural basis for its catalytic function is unknown. When cultured in media containing extensively purified apple RG-II as a sole carbon source, a *B. thetaiotaomicron* genetic mutant lacking the BT1017 enzyme (Δ BT1017) produces a pentasaccharide Rha- α 1,3-Api- β 1,2-(Araf- α 1,3)-(6-O-Me-GalA- α 1,4)-GalA here referred to as Δ BT1017oligoA (Fig. 1A) (5). The complete degradation of Δ BT1017oligoA requires five enzymes (BT1017, BT1018, BT1021, BT1012, and BT1001) collectively referred to here as A5 (Fig. 1B). BT1017 cleaves the 6-O-methylester linkage from the backbone GalA; BT1018 (α -galacturonidase) cleaves the glycosidic linkage between the two backbone GalA residues; BT1021 (α -arabinofuranosidase) cleaves the linkage between Araf (chain F) and the reducing end GalA; BT1012

This article contains supporting information.

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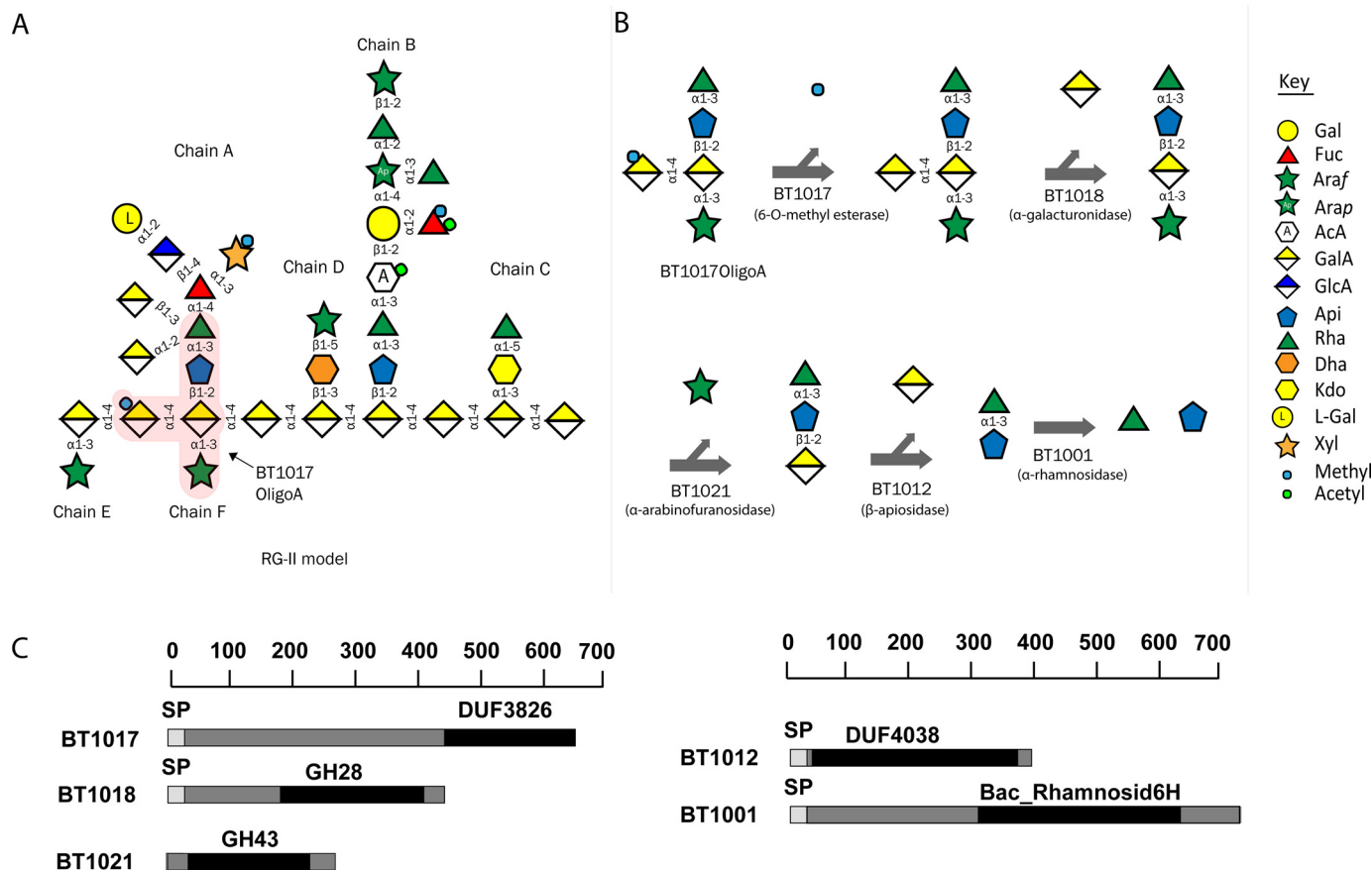


Figure 1. Structure and degradation of pectin RG-II. **A**, structural model of RG-II showing various side chains and Δ BT1017oligoA (pink shading). **B**, pathway showing the five enzymes (A5) required for complete Δ BT1017oligoA degradation in the human gut microbe *B. thetaiotaomicron*. Gal, galactose; Fuc, fucose; Araf, arabinofuranose; Arap, arabinopyranose; AcA, aceric acid; GalA, galacturonic acid; GlcA, glucuronic acid; Api, apiose; Rha, rhamnose; Dha, 3-deoxy-D-lyxo-2-heptulosaric acid; Kdo, 3-deoxy-D-manno-2-octulosonic acid; L-Gal, L-galactose; Xyl, xylose; Methyl, methyl group; Acetyl, acetyl group. **C**, modular architecture of BT1017 and various proteins involved in Δ BT1017oligoA degradation.

(β -apiosidase) cleaves the linkage between Api and the reducing end GalA; and finally BT1001 (α -rhamnosidase) cleaves the linkage between Rha and Api in the Rha–Api disaccharide (Fig. 1B).

In the present study, we showed that the *B. thetaiotaomicron* mutant Δ BT1017, when cultured in carbohydrate extract from apple juice (CEAJ) as a sole carbon source, generates a second oligosaccharide (hereafter referred to as Δ BT1017oligoB). Our structural analyses revealed that Δ BT1017oligoB is a dimethylated nonasaccharide containing components of the RG-II backbone and its side chains. We characterized the kinetic properties of BT1017, showing that the enzyme has a low turnover against apple RG-II, Δ BT1017oligoA, and Δ BT1017oligoB and hence may represent a rate-limiting step during RG-II metabolism. We revealed that BT1017 is a serine esterase with an α/β -hydrolase fold and hence has not evolved from the progenitor protein that gave rise to the CE8 family of PMEs, which are predominantly comprised of right-handed β -helices.

Results

Characterization of Δ BT1017oligoB

B. thetaiotaomicron Δ BT1017 deletion mutant was cultured on CAJ for 48 h to stationary phase ($A_{600\text{ nm}} \sim 1.0$), and TLC was first used to analyze the culture supernatants. The data showed that Δ BT1017 generates two oligosaccharides, defined

as Δ BT1017oligoA and Δ BT1017oligoB (Fig. 2A). Both sugars were purified by size-exclusion chromatography and treated independently with a mixture of recombinant A5 enzymes including BT1017, BT1018 (α -galacturonidase), BT1021 (α -arabinofuranosidase), BT1012 (β -apiosidase), and BT1001 (α -rhamnosidase), which target specific glycosidic linkages in RG-II (5). Unless otherwise stated, all the recombinant RG-II-degrading enzymes mentioned in this text were the same constructs used by Ndeh *et al.* (5) and lack the N-terminal signal peptide (SP) regions (Fig. 1C). The products of the enzymatic treatment were then analyzed by TLC and HPLC (Fig. 2, B and C). Digestion of Δ BT1017oligoA yielded GalA, Araf, Rha and Api indicating that the molecule is the methylated pentasaccharide (Rha- α 1,3-Api- β 1,2-(Ara- α 1,3)(6-O-Me-GalA- α 1,4)-GalA, which was used to demonstrate the site of action of the PME in Ndeh *et al.* (5). The digestion of Δ BT1017oligoB, on the other hand, was incomplete, yielding only two monosaccharides (Araf and GalA) and a third product of unknown identity (Fig. 2, B and C). The release of GalA and Araf from Δ BT1017oligoB by Δ BT1017oligoA-specific enzymes BT1018 (α -galacturonidase) and BT1021 (α -arabinofuranosidase) suggests that Δ BT1017oligoB contains the backbone GalA and the side-chain F Araf sugars characteristic of Δ BT1017oligoA (Fig. 1, A and B).

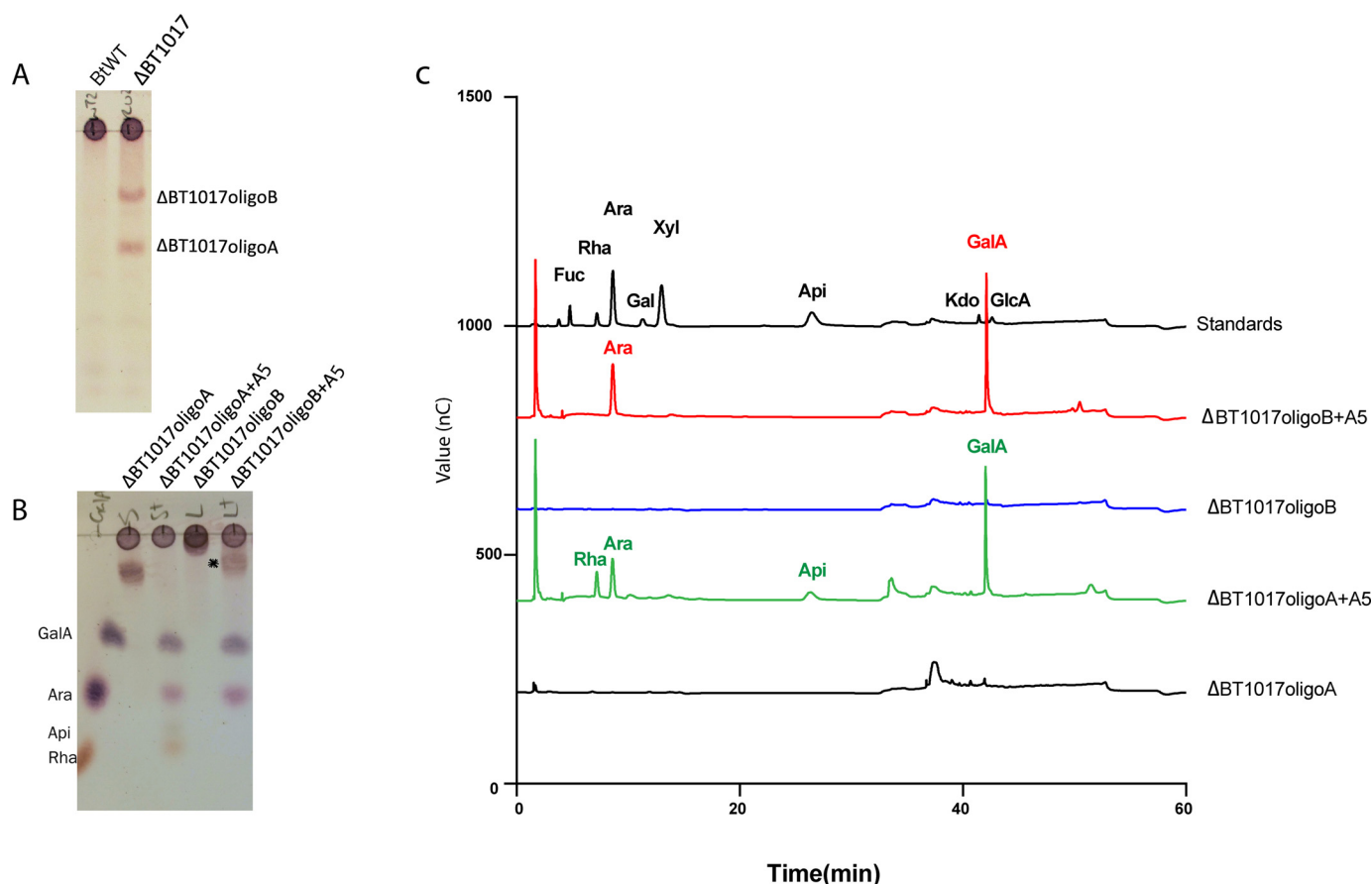


Figure 2. *B. thetaiotaomicron* deletion mutant Δ BT1017 generates two oligosaccharides, Δ BT1017oligoA and Δ BT1017oligoB, during growth on CEAJ. **A**, TLC analyses of culture supernatants from *B. thetaiotaomicron* WT (BtWT) and Δ BT1017 cells post-growth on CEAJ. **B**, digestion of Δ BT1017oligoA and B with a mixture of Δ BT1017oligoA-degrading enzymes (A5) containing BT1017, BT1018, BT1021, BT1012, and BT1001. Each substrate was treated with A5, and the reaction was stopped and analyzed by TLC. The product marked with an asterisk is the leftover of Δ BT1017oligoB after digestion with the mixture of A5 enzymes. **C**, HPLC analyses of samples in Δ BT1017oligoB.

To determine the full structure of Δ BT1017oligoB, a combination of MS, enzymatic and NMR analyses were performed. First, MS data revealed that Δ BT1017oligoB has a protonated molecular mass ($[M + H]^+$) of 1453.44 Da (Fig. 3A). When treated with BT1017, the mass of Δ BT1017oligoB decreased by 28.03 Da (Fig. 3B), corresponding to the loss of two methyl groups. This suggests that Δ BT1017oligoB contains two ester-linked methyl groups that were hydrolyzed by the BT1017 PME. Second, when WT *B. thetaiotaomicron* was cultured on Δ BT1017oligoB, the bacterium accumulated the disaccharide 2-O-Me-Xyl- α 1,3-Fuc, which is unique to side chain A of RG-II but not present in Δ BT1017oligoA (Fig. 3C). The sugar Api was also detected. These results demonstrate that Δ BT1017oligoB contains components of Δ BT1017oligoA and additional sugars from RG-II side chain A. Last, Δ BT1017oligoB was shown to be susceptible to attack by the β -D-glucuronidase enzyme BT0996, which released GlcA (Fig. 3D). Because this required pretreatment with BT1017, this result suggests that at least one of the methyl decorations in Δ BT1017oligoB sterically hinders the activity of BT0996. Release of free GlcA is also an indication that Δ BT1017oligoB lacks the terminal L-Gal residue, which is α 1,2-linked to GlcA at the nonreducing end of chain A (Fig. 1A).

Based on the above features of Δ BT1017oligoB (protonated mass of 1452.44 Da, presence of 2-O-Me-Xyl- α 1,3-Fuc, GalA,

Ara, Api, and GlcA (highlighted in Fig. S1A) and the absence of terminal L-Gal, two possible structures of de-methyl-esterified BT1017oligoB (Δ BT1017oligoB-2Me) were deduced from the known structure of RG-II (Fig. S1, B and C). These include Δ BT1017oligoB-2Me- α (GlcA- β 1,4-(2-O-Me-Xyl- α 1,3)-Fuc- α 1,4-(GlcA- α 1,2)-Rha- α 1,3-Api- β 1,2-(Araf- α 1,3)-(GlcA- α 1,4)-GalA) and Δ BT1017oligoB-2Me- β (GlcA- β 1,4-(2-O-Me-Xyl- α 1,3)-Fuc- α 1,4-(GlcA- β 1,3)-Rha- α 1,3-Api- β 1,2-(Araf- α 1,3)-(GlcA- α 1,4)-GalA).

Both sugars differ by the presence of either α 1,2- or β 1,3-linked GalA (underlined). To determine which of them corresponded to Δ BT1017oligoB-2Me, enzymes targeting all linkages in the predicted sugars (Δ BT1017oligoB-2Me- α and Δ BT1017oligoB-2Me- β) were used to sequentially digest Δ BT1017oligoB. The first set of recombinant enzymes collectively referred to here as B5 enzymes include BT1017, BT1018, BT1021, BT0996, and BT1012. These together should cleave the two ester groups, the backbone α 1,3-linked GalA, the side chain F α 1,3-linked Araf, the side chain A β 1,4-linked GlcA, and the reducing end/backbone GalA residue, respectively (Fig. S1, D and E), to generate two possible pentasaccharide structures: MXFGRA- α (2-O-Me-Xyl- α 1,3-Fuc- α 1,4-(GlcA- α 1,2)-Rha- α 1,3-Api) and MXFGRA- β (2-O-Me-Xyl- α 1,3-Fuc- α 1,4-(GlcA- β 1,3)-Rha- α 1,3-Api), differing by the presence or

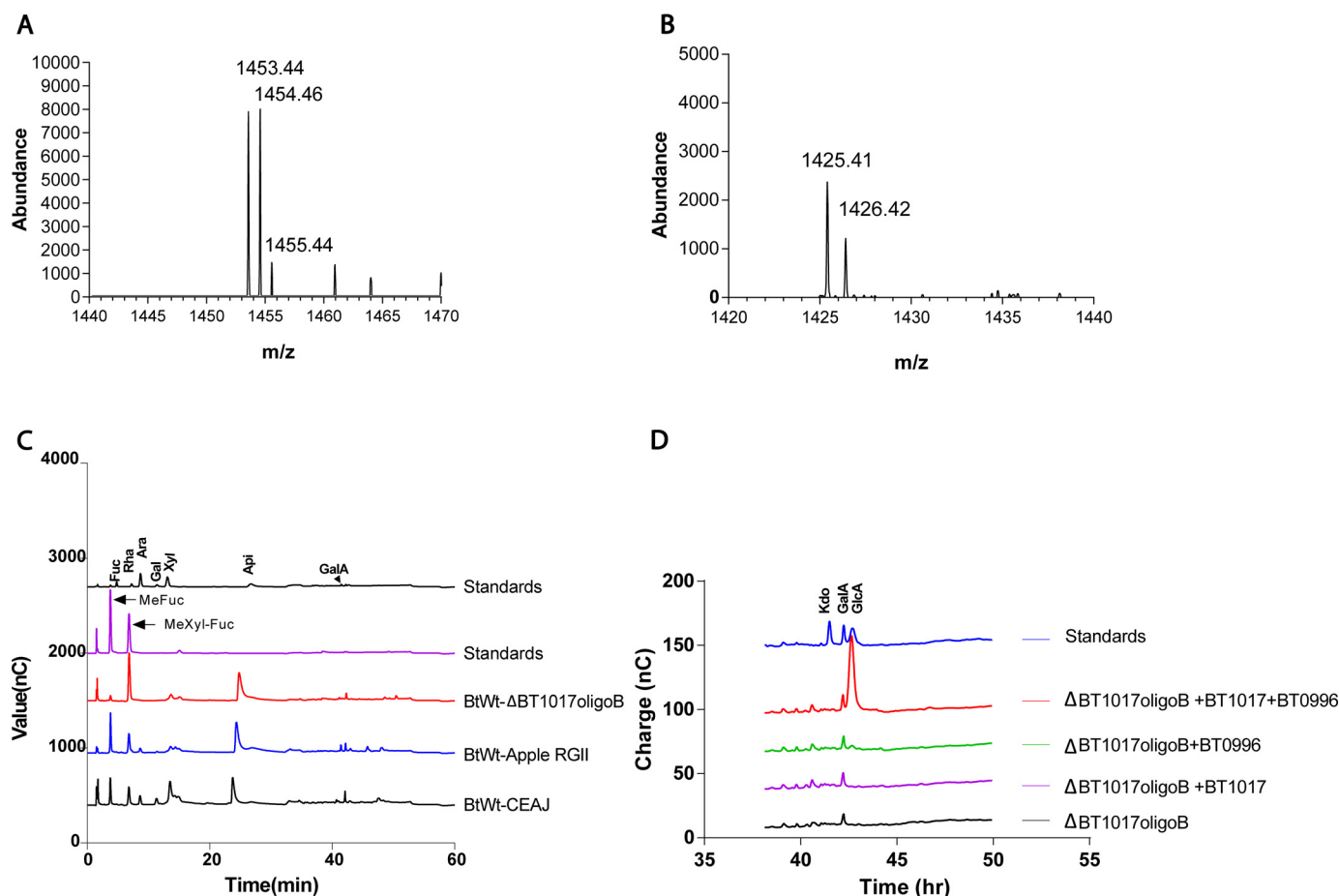


Figure 3. Analyses of Δ BT1017oligoB by MS and HPLC. A, MS of purified Δ BT1017oligoB. Masses presented are for Δ BT1017oligoB and fragments plus hydrogen ions $[H]^+$. B, MS of purified Δ BT1017oligoB after digestion with BT1017. C, HPLC analyses of spent media following growth of *B. thetaiotaomicron* Δ BT1017 on Δ BT1017oligoB, apple RG-II, and CEAJ. MeFuc: 2-O-Me-Fuc, MeXyl-Fuc: 2-O-Me-Xyl- α 1,3-Fuc. D, HPLC data showing activity of BT0996 and BT1017 on Δ BT1017oligoB.

absence of either α 1,2- or β 1,3-linked GalA (underlined). Digestion of Δ BT1017oligoB-2Me with a mixture of recombinant B5 enzymes (BT1017, BT1018, BT1021, BT0996, and BT1012) and subsequent analyses by TLC revealed the generation of a product that migrates to a similar extent as the sugar standard MXFGRA- β (Fig. 4A, lane 8 in white rectangle). However, it was also possible that the product corresponded to MXFGRA- α because of the significant structural similarity to MXFGRA- β . As a result, it was referred to as MXFGRA-x. Both α 1,2- and β 1,3-GalA linkages in MXFGRA- α and MXFGRA- β have been shown to be specifically targeted by the enzymes BT0997 (α -galacturonidase) and BT0992 (β -galacturonidase), respectively (5) (Fig. S1, D and E); hence to determine whether the product contained α - or β -linked GalA residues, each of these enzymes (BT0992 and BT0997) was used to further digest MXFGRA-x. TLC analyses of the reaction showed that MXFGRA-x was digested by BT0992 but not by BT0997 (Fig. 4A, lanes 9 and 10, respectively), indicating that the exposed GalA residue in the product was β 1,3-linked to Rha and that the pentasaccharide was MXFGRA- β . This was also confirmed by 2D HSQC NMR analyses of Δ BT1017oligoB, which detected 1H and ^{13}C HSQC anomeric signals (δ_H 4.67 and δ_C 104.1) of β -GalA. The NMR analyses also revealed H1/C1 signals of all other carbohydrate residues in the anomeric

region of the spectrum. These include signals for GalA- α 1-4, AraF- α 1-3, Api- β 1-2, Rha- α 1-3, Fuc- α 1-4, 2-O-Me-Xyl- α 1-3, and GlcA- β 1,4 (Fig. 4B), which were assigned by comparison with published data (10). Two weaker cross-peaks could be assigned to the anomeric center of the reducing-end GalA residue in the backbone of Δ BT1017oligoB. The full monosaccharide composition of Δ BT1017oligoB-2Me was confirmed by treatment of the sugar with a combination of A5 enzymes together with BT0996, BT0992, and other RG-II-degrading enzymes BT1002 (α -L-fucosidase) and BT1001 (α -L-rhamnosidase) and analyses of the digested sample by HPLC. The results showed that the enzymes degraded the sugar into all its constituent monosaccharides GlcA, GalA Rha Api AraF, and the disaccharide 2-O-Me-Xyl- α 1,3-Fuc (Fig. 4C). A model showing the cleavage sites of various enzymes on Δ BT1017oligoB is shown in Fig. 4D.

Activity of BT1017 and kinetic parameters

Full-length BT1017 (BT1017-FL) is a 73.7-kDa protein consisting of a SP (positions 1–20), a sequence of unknown function (positions 20–400), or central module (CM) and a domain of unknown function DUF3826 (positions 400–600) (Figs. 1 and 5). To determine the site of the esterase activity in BT1017,

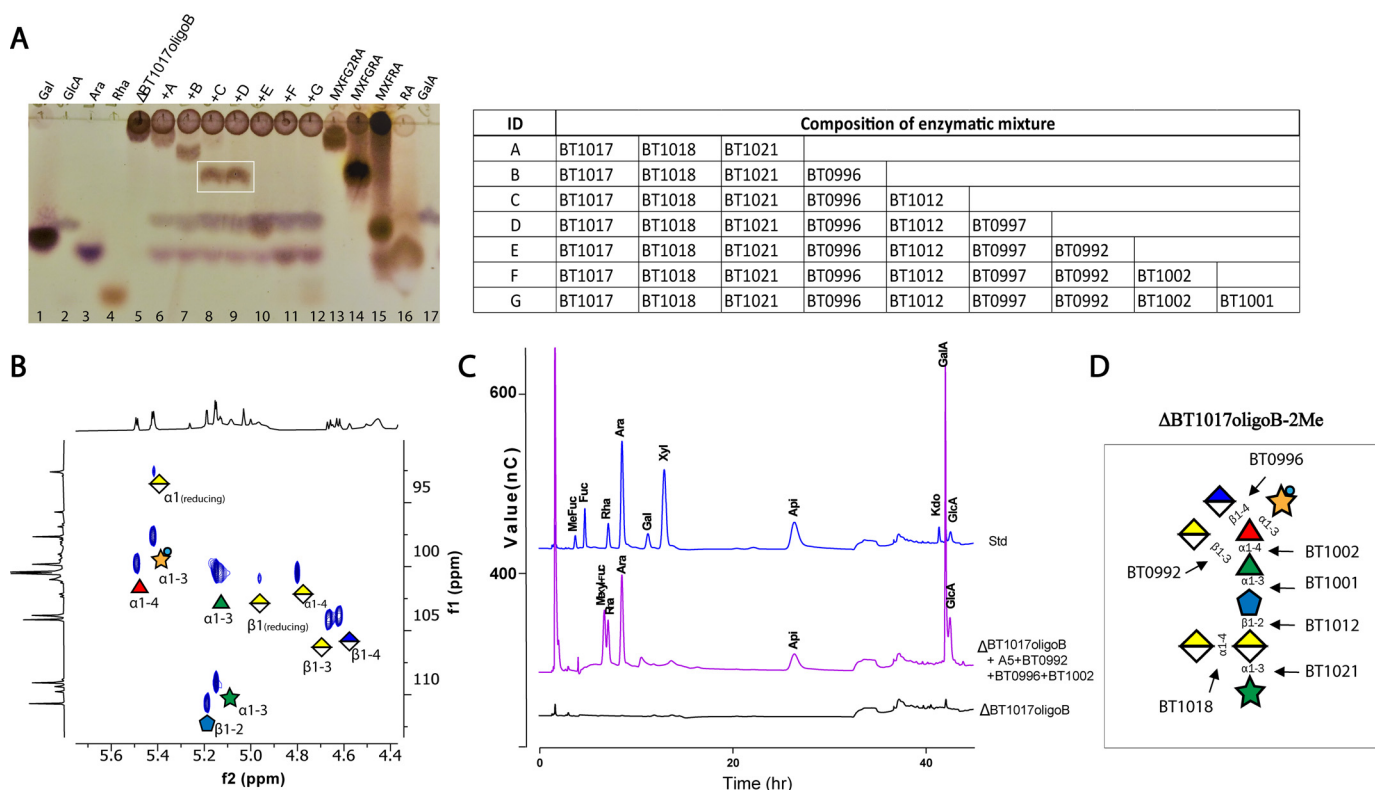


Figure 4. Structural characterization of Δ BT1017oligoB. A, TLC analysis Δ BT1017oligoB after digestion with diverse combinations of RG-II-degrading enzymes. The bands in the white rectangle correspond to MXFGRA- β later confirmed to be MXFGRA- β (2-O-Me-Xyl- α 1,3-Fuc- α 1,4-(GalA- β 1,3)-Rha- α 1,3-Api). Other complex sugar standards include MXFG2RA (2-O-Me-Xyl- α 1,3-Fuc- α 1,4-(GalA- α 1,2)(GalA- β 1,3)-Rha- α 1,3-Api), MXFRA (2-O-Me-Xyl- α 1,3-Fuc- α 1,4-Rha- α 1,3-Api), and RA (Rha- α 1,3-Api). B, HSQC NMR of anomeric region of Δ BT1017oligoB. C, HPLC analyses of Δ BT1017oligoB after complete hydrolysis with a mixture of Δ BT1017oligoB-degrading enzymes. D, model of Δ BT1017oligoB-2Me showing the cleavage sites of various Δ BT1017oligoB-degrading enzymes.

various recombinant fragments of the protein namely BT1017-SP (71.7 kDa; lacking the signal peptide), BT1017-CM (49.4 kDa; corresponding to CM), and BT1017-DUF3826 (23.5 kDa; corresponding to the DUF3826 domain) (Fig. 5) were expressed and tested against apple RG-II using a coupled spectrophotometric enzyme assay that measures released methanol (as described earlier (11)). Only BT1017-SP and BT1017-CM showed activity against the substrate (Table 1), indicating that BT1017-CM comprises the catalytic site. The optimal temperature and pH for BT1017 activity were determined to be 37 °C and 8.5, respectively (Fig. S2). To further explore the specificity of the enzyme, BT1017-SP was tested against various methyl-

and acetyl-esterified substrates. The enzyme was active against Δ BT1017oligoA, Δ BT1017oligoB, 6-O-methyl galacturonic (Me-GalA), and 6-O-methyl glucuronic acid (Me-GlcA) but not methylpropionate, methylbutyrate, ethylpropionate ethylbutyrate, acetylated potato RG-I, and 4-nitrophenyl-acetate (Table 1). BT1017 thus appears to have a preference for methylated hexose sugars. The K_m and k_{cat} of BT1017-SP and BT1017-CM against various substrates are reported in Table 1, showing that BT1017-SP has an \sim 2-fold higher catalytic efficiency compared with BT1017-CM against more complex substrates such as apple pectin. BT1017-SP also showed a higher k_{cat}/K_m toward Me-GalA and Δ BT1017oligoB compared with Me-GlcA and Δ BT1017oligoB; however, the difference was less than 10-fold.

3D structural features of BT1017

BT1017-SP could not be crystallized. BT1017-CM, however, generated crystals in the space group C2221 with one molecule in the asymmetric unit. The structure of the enzyme was determined by single-wavelength anomalous dispersion phasing and refined to 1.9 Å with an R_{factor} of 20.04% and an R_{free} of 24.09%. The final model (PDB entry 6GOC) of BT1017-CM consists of residues 20–462 of BT1017. The stereochemical quality of the model was assessed by validation tools in Coot and MolProbity (12, 13). The ratios of preferred and allowed regions from the Ramachandran plot are 96.61 and 3.17%, respectively. The statistics for data collection and refinement are summarized in





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